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Guidelines for using *in vitro* methods to study the effects of phyto-oestrogens on bone

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These guidelines review the relevant literature on the way plant phyto-oestrogens act on bone and the responsiveness of different bone cell systems to phyto-oestrogenic compounds. The primary emphasis is on the experimental conditions used, the markers available for assessing osteoblast and osteoclast function, and their expected sensitivity. Finally, we assess the published results to derive some general recommendations for *in vitro* experiments in this area of research.

Phyto-oestrogens: Osteoblast: Osteoclast: Osteoprogenitor bone marrow cells

Introduction

It has often been suggested that the consumption of dietary phyto-oestrogens may have beneficial effects on bone health at all stages of life. But the experimental evidence supporting these assertions is often equivocal or less than definitive. Additional research is therefore essential to determine how phyto-oestrogens act on bone cells, and so obtain a more complete understanding of the effects of phyto-oestrogens on the skeleton. Before reviewing the experimental evidence and procedures, however, we first summarise the mechanisms that could be involved in mediating the action of phyto-oestrogens.

Mechanisms of action

Because phyto-oestrogens are likely to act in several ways, their effects on other molecules, cells and tissues must be considered. Their most relevant molecular actions are those mediated by oestrogen receptors (ER), but they may have other actions that are ER-independent. Phyto-oestrogens act at all stages of the cell cycle, including apoptosis, and they may well change the response to cytokines and growth factors. The available evidence for these actions is reviewed briefly.

Molecular actions

Interaction of phyto-oestrogens with oestrogen receptors. The rat, mouse and human ER exist as two subtypes, ERα and ERβ, which differ in the C-terminal ligand-binding domain and in the N-terminal transactivation domains (Paech *et al.* 1997). While cells of the osteoblast lineage and chondrocytes bear ER, their presence in osteoclasts is not yet entirely clear. Preosteoclasts bear detectable concentrations of ERα, but osteoclast maturation and bone resorption are associated with the loss of ERα (Oreffo *et al.* 1999). ERα is significant for bone metabolism. For example, a 28-year-old man was found to have a disruptive mutation in his ERα gene. This caused oestrogen resistance and resulted in decreased bone mineral density, increased bone turnover and incomplete epiphyseal closure (Smith *et al.* 1994). A second ER subtype, ERβ, has recently been cloned. This suggests that there may be alternative pathways for mediating oestrogen actions. Both ERα and ERβ have been detected in many human tissues, but often at different concentrations, implying distinct physiological roles for each of them (Kuiper *et al.* 1997). The patterns of the two ER are also different during human osteoblast differentiation (Arts *et al.* 1997). *In vitro* experiments indicate that the phyto-oestrogens have different binding affinities for ERα

Abbreviations: ER, oestrogen receptor; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; M-CSF, macrophage-colony stimulating factor; PTH, parathyroid hormone; PTK, protein tyrosine kinase; RANK, receptor activator of NF-κB; RANK-L, receptor activator of NF-κB ligand; TGF-β, transforming growth factor-β; TRAP, tartrate-resistant acid phosphatase; VENUS, Vegetal Estrogens in Nutrition and the Skeleton.

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and ERβ, with some compounds, such as coumestrol, binding more strongly to ERβ than to ERα, and more avidly than 17β-oestradiol (Kuiper *et al.* 1997).

Recent detailed structural analyses of the ER have revealed that they undergo distinct conformational changes when they bind various ligands; these may lead to a spectrum of agonist or antagonist interactions. Thus, genistein binds to ERβ in a manner similar to 17β-oestradiol but the transactivation helix does not adopt the proper 'agonist' position, so that genistein is classified as a partial agonist (Pike *et al.* 1999). Studies with a series of synthetic drugs designed to modify ER action selectively show that the phyto-oestrogens can have a very wide range of partial agonist/antagonist actions when they bind to ER (Barkhem *et al.* 1998).

Alternative, non-classical mechanisms. Phyto-oestrogen-like compounds may have rapid effects by interacting with enzymes involved in signal transduction, such as protein tyrosine kinase (PTK) and mitogen-activated protein kinase, or with the DNA-modifying enzyme, topoisomerase II. Oestrogen stimulates the activity of mitogen-activated protein kinase in a rat bone cell line at concentrations 100-fold lower than those required for ER-mediated transcriptional activation (Endoh *et al.* 1997). Genistein has been used widely to inhibit PTK, and this may be responsible for some of its effects on bone cells. The situation is, however, complex as genistein and another PTK inhibitor, tyrophostin A51, have very different effects on cell proliferation and differentiation of normal, human bone cell lines (Yoon *et al.* 1998).

In addition to inhibiting PTK, genistein also inhibits DNA topoisomerase II, with a marked effect on cell proliferation and transformation (Markovits *et al.* 1989). Genistein inhibits the decatenation activity of the isolated enzyme *in vitro*, and stimulates topoisomerase-mediated double-strand breaks in DNA. This action of genistein seems to be relatively specific, as five other structurally related compounds had no similar activities (Markovits *et al.* 1989). The action of genistein has been associated with the induction of apoptosis in some cell types (McCabe & Orrenius, 1993), but more recent data suggest that topoisomerase-mediated DNA damage and apoptosis are unconnected, at least in colon cancer cells (Salti *et al.* 2000).

Cells and tissues

Although the actions of phyto-oestrogens on cells and tissues may well include many of the molecular events discussed above, the evidence for a precise action is often lacking, primarily because of the complex interrelationships between the processes involved.

Effects on bone cell function. Oestrogen-like compounds may well affect a wide range of bone cell functions, including proliferation, differentiation and cell death (apoptosis). Evidence has accumulated that the major effect of oestrogens on bone remodelling is to decrease bone resorption. A lack of 17β-oestradiol caused by early menopause or ovariectomy results in accelerated bone resorption, and oestrogen replacement therapy can delay or prevent this condition in women.

Hughes *et al.* (1996) showed that oestrogens cause osteoclasts to undergo apoptosis through increased production of transforming growth factor-β1 (TGF-β) by osteoblasts. TGF-β increases the production of osteoclastogenesis inhibitory factor (now referred to as osteoprotegerin; American Society for Bone and Mineral Research President's Committee on Nomenclature, 2000) mRNA in primary osteoblasts and in osteoclastic stromal cells lines, and inhibits the number of surviving osteoclasts in mouse bone marrow culture (Murakami *et al.* 1998). Further studies have provided a much more complete picture of the interactions between stromal and osteoclastic cells to control osteoclast formation through the action of members of the tumour necrosis factor family of receptors and ligands, as discussed below (see 'Osteoclast formation and bone resorption studies').

The insulin-like growth factors (IGF-I and IGF-II) promote the proliferation of many cell types and play a key role in cell cycle progression, cell proliferation and tumour progression (Mohan & Baylink, 1991). Most of the effects of IGF are mediated by binding to the type 1 IGF receptor, whereas the type 2 IGF receptor is mainly involved in the clearance of IGF-II (Mohan & Baylink, 1993). The IGF in all biological fluids are bound to at least six different high-affinity binding proteins (IGFBP 1–6). The IGFBP prolong the IGF half-life, counteract the insulin-like hypoglycaemic effect of IGF, maintain a reservoir of IGF in the circulation, transport IGF from the circulation to peripheral tissues, modulate IGF action, and also exert IGF-independent actions (Schmid, 1995). The complexity of the IGF system is further increased by the intervention of specific proteases, which, by fragmenting IGFBP, reduce their affinity for IGF and eventually lead to greater IGF bioavailability (Rajaram *et al.* 1997). Several new lines of investigation indicate that IGF-I is important in the skeleton. IGF-I enhances osteoblast differentiation, helps to maintain the osteoblast phenotype, and inhibits collagenase activity (Mohan & Baylink, 1993). IGF-I may also recruit premature osteoclasts, and serve as a coupling agent in the bone remodelling cycle (Mochizuki *et al.* 1992). All six IGFBP are produced by bone cells, and modulate IGF-mediated osteoblast proliferation and differentiation (Jones & Clemmons, 1995). IGFBP-3 triggers apoptosis and mediates the effects of TGF-β on apoptosis (Rajah *et al.* 1997). Furthermore, the type V TGF-β receptor may be the putative IGFBP-3 receptor. Oestrogens also increase the proteolytic activity of IGFBP-4 in SaOS-2 cells (osteoblast-like cells), leading to increased IGF bioactivity and IGF-I-mediated cell proliferation (Kudo *et al.* 1996). Thus, phyto-oestrogens seem to act like oestrogens on bone cells, affecting the IGF system. They increase the proteolysis of IGFBP-4, so making IGF more available, leading to osteoblast proliferation. Osteoblasts may, in turn, produce TGF-β, which may induce osteoclast apoptosis, either directly or via IGFBP-3, thus protecting the body from bone loss and osteoporosis.

Effect on acidification capacity. Osteoclastic acid secretion is regulated by the phosphorylation of cell membrane components. Osteoclasts are unusually dependent on $pp^{60c\text{-}src}$, the product of *c-src* gene, which is required for

bone resorption (Soriano *et al.* 1991; Boyce *et al.* 1992). Although PTK often modulate gene expression or cell differentiation, the concentration of $pp^{60c\text{-}src}$ in the cell membrane of end-differentiated avian osteoclasts suggests that it has a more directed role in modulating cellular activity, possibly via regulating the function of existing membrane proteins (Williams *et al.* 1998). Some phyto-oestrogens, such as genistein, are PTK inhibitors, and the similarities between the dose response for kinase inhibition and the decrease in membrane acid transport suggest that this inhibitory action is responsible for the membrane effect. Phyto-oestrogens may also act on osteoclast formation by inhibiting macrophage-colony stimulating factor (M-CSF), a cytokine required for osteoclastogenesis, which is inhibited by oestrogens (Srivastava *et al.* 1998).

Effects on cell adhesion molecules. As steroid hormones, including 17β-oestradiol, regulate the synthesis in endocrine tissues of cell adhesion molecules, these proteins may be targets for phyto-oestrogens. Osteoblast cells contain a repertoire of cell adhesion molecules (Cheng *et al.* 1998), which appear to be essential for maintaining anti-apoptotic signals. The catenins (α, β, γ) are intracellular proteins that link the cytoplasmic domains of the cadherins to the actin-based cytoskeleton and act as transducers of intracellular signals. A relatively high concentration of genistein causes apoptosis of osteoblasts *in vitro*, via the activation of caspase-3 and the specific cleavage of cadherins and catenins (Hunter *et al.* 2001). Phyto-oestrogens may therefore act in bone and other tissues to influence cellular interactions.

Bone formation assays

Bone consists of various types of cells and an abundant mineralised extracellular matrix. Bone formation and bone resorption are essential processes for normal bone morphogenesis and Ca homeostasis. The formation of endosteal bone is a complex process, which depends on the proliferation of osteoprogenitor cells in the marrow stroma, the differentiation of preosteoblasts into mature osteoblasts, and the activity of differentiated osteoblasts. Each of these steps is essential for the initiation of osteogenesis and the deposition of new bone matrix in the remodelling unit. Histomorphometric studies indicate that bone formation is mainly regulated by the proliferation of osteoprogenitor cells and the activity of mature osteoblasts. The rate of bone formation also depends on the number of osteoblasts rather than on their activity (Baylink & Liu, 1979).

This part of the review updates the information available on bone formation assays and discusses new systems and methods for studying bone mineralisation. Most bone culture systems use long bones or calvaria from fetal/neonatal rats and mice (Stern & Raisz, 1979), but recently developed systems have used cultures of dispersed osteoblast and bone marrow cells, which mineralise *in vitro*. Several bone induction models, such as the implantation of osteoblast-like cells in open and closed systems, have been used to demonstrate their capacity to form bone. These types of bone-forming systems are also reviewed. As the central process in endochondral bone formation is the progressive differentiation of proliferating chondrocytes to growth-arrested hypertrophic cells that direct the mineralisation of the cartilage matrix and its subsequent replacement by bone (Cancedda *et al.* 1995), we also examine the various culture systems used for chondrocytes. Lastly, a number of studies using osteocytes in culture have suggested that osteocytes play an important role in bone metabolism, especially in its regulation by mechanical stimuli (Cowin *et al.* 1991).

Bone organ culture

Origin of the bone. Bone formation assays use tissues from various sources (Baylink & Liu, 1979), and may be fetal, newborn or even adult bone. Chicken, mouse and rat bone cultures are the most common, although human bone fragments have also been used. Bones from calvaria and limbs are most frequently cultured. Fetal bone, because of its thinness, allows better diffusion of nutrients in culture. Fetal calvaria have the advantage of forming only intramembranous bone without a cartilage template. However, the growth of long bones is mainly endochondral. Endochondral and intramembranous bone may respond differently to hormones, growth factors and environmental conditions, since the calvarium is not subjected to mechanical forces. Therefore, most *in vitro* organ culture systems have limitations, but the models available provide important information on the processes involved in bone formation.

Organ culture systems. Several bone organ culture systems that mineralise *in vitro* have been developed. The source of the bone, the media and the culture conditions all affect the sensitivity of bones to hormones (Soskolne *et al.* 1986). Mouse long bones have predominantly been used. The growth of long bone, as measured by diaphyseal length and Ca and P uptake, depends on the age of fetal long bone (Soskolne *et al.* 1986). Fetal mouse long bones are capable of forming bone *in vitro* and becoming mineralised, depending on the type of medium used and the supplements added (Soskolne *et al.* 1986; Dieudonne *et al.* 1994).

Cultures of 20 d fetal rat parietal bones have been used to study bone formation in well-defined, serum-free systems (Gronowicz *et al.* 1989; Lee *et al.* 1992*a*), while 21 d fetal rat calvaria have been used to study the effect of hormones and growth factors on collagen synthesis and cell proliferation (Rowe & Kream, 1982; Kream *et al.* 1990). However, fetal rat calvaria, including parietal and frontal bones, fail to mineralise *in vitro*. This may be due to the presence of large cartilaginous and fibrous segments (sutures), which separate the frontal bone from the parietal bone. Studies in which the periosteum was stripped from the central part of the calvarium showed how several hormones affected bone formation, and identified the cell target (Rowe & Kream, 1982; Chyun *et al.* 1984).

Mouse calvaria can also form bone *in vitro*, but their responses differ from those of fetal rat calvaria and long bones. The periosteum of neonatal mouse calvarium is thinner than that of fetal and neonatal rat calvaria, which may contribute to the differences in their responses.

Cultures of chick embryos *in vitro* are excellent for studies on bone formation, especially the effects of Ca

deficiency (Jacenko & Tuan, 1986). Tibiae from 8-d-old embryonic chicks have been also used (Howard *et al.* 1980). Folded periostea from the calvaria of 17-d-old embryonic chicks mineralise *in vitro* (Tenenbaum & Heersche, 1986). The periosteum must be folded to create a microenvironment in which osteoblasts can develop and mineralise.

Few studies have used human bone fragments. The responses of human bone to hormones are generally similar to those of fetal rat bone (Keck *et al.* 1984).

Quantitative methods for the evaluation of bone formation. Collagen and DNA synthesis, calcification and bone morphology should all be tested in order to assay bone formation in culture.

The type of collagen should be determined first. Since osteoblasts predominantly synthesise type I collagen, the proportions of type I, II and III collagen should be assessed. The proportions of type I and type III collagen can be measured by interrupted gel electrophoresis (Sykes *et al.* 1976). Molecular probes are also available for most of the different types of collagen, and Northern blot analysis is suitable for determining the relative proportions of collagen mRNA. Once type I collagen has been shown to be the predominant collagen, the incorporation of [^{3}H]proline can be used to assay bone formation (Peterkofsky & Diegelmann, 1971; Chen & Raisz, 1975).

DNA synthesis may be measured by labelling bones with methyl [^{3}H]thymidine for their last 2 h in culture (Gronowicz *et al.* 1994). The DNA content can be measured by fluorimetry (Labarca & Paigen, 1980).

The measurement of Ca in cultured bone is an important indicator of bone formation *in vitro*. A colorimetric assay with *o*-cresolphthalein is commonly used to measure calcification in TCA extracts of cultured bones (Gronowicz *et al.* 1989). Calcein, a fluorescent dye that stains calcium phosphate deposits (Hock *et al.* 1968), can be used to measure calcification in mineralising cell cultures. Calcification, however, can be increased by bone damage or death (Ramp & Neuman, 1971). Therefore, the bone should also be checked by histological examination.

Several histological methods can be used to assess bone morphology (Malluche & Faugere, 1986). The following parameters may be evaluated: bone volume, trabecular thickness, osteoid thickness, osteoblast and osteoclast surface areas and mineral apposition rate.

Osteoblast cell culture systems

The isolation and culture of bone-derived cell populations have greatly enhanced our ability to understand the factors that influence the proliferation and differentiation of bone cells. We review selected aspects of bone cell culture relating to the osteoblast lineage, such as the basic experimental methods used to culture osteoblast cells and the types of cell culture systems currently used to study osteoblast biology. This is followed by a brief discussion of the issues faced by investigators using these systems. Several recent articles have also discussed various osteoblast cell culture models and provide some critical commentaries about their use (Marie, 1994; Rodan *et al.* 1994; Gundle & Beresford, 1995; Parfitt, 1995; Robey, 1995).

General aspects. The many and varied osteoblast culture systems that have been developed include cultures containing osteoblast or osteoblast-like cells from different species, bones of different ages, and a variety of anatomical sites and pathological states. Systems have also been developed for specific cell populations, such as osteoprogenitor cells and osteocytes. Despite the diversity in these systems, most have many features in common. All of the systems currently used have very similar basic culture environments (culture medium composition, serum type and concentration, temperature, atmosphere, antibiotics) and methods of maintenance (feeding, sub-culturing, cloning). The methods used to release cells from bone are also based on two classic techniques: proteolytic digestion and explant outgrowth. Some basic techniques of bone initiation and culture as well as phenotype assays are available (Sodek & Berkman, 1987).

Most cell culture systems have used one of two distinct approaches: in one the cells were used soon after removal from the tissue (i.e. after a minimal number of subcultures); in the other, the cells have been established as permanent cell lines. Phenotype changes, such as dedifferentiation, cellular senescence or overgrowth by the more proliferative cell types, are limited in short-term cultures. Therefore this type of osteoblast culture may reflect many of the properties of osteoblasts in tissues (Fedarko *et al.* 1995). However, the range of cell types in this culture system will depend on the method of isolation, and the presence of multiple cell types cannot be ruled out since this technique does not involve cloning. The results obtained with such cell cultures are, however, consistent and highly reproducible (Robey, 1995). Although the relationship between phenotype stability and cellular heterogeneity is still unclear (Nijweide *et al.* 1981; Rodan & Rodan, 1984; Owen *et al.* 1990), at least some osteoblast characteristics may be retained over numerous passages of mixed bone cell populations (Schmidt & Kulbe, 1993). It is also clear that even osteoblast cells derived by cloning can be heterogeneous in their expression of osteoblast traits and developmental potential (Grigoriadis *et al.* 1985; Yamaguchi & Khan, 1991). The phenotypes of osteosarcoma cell lines derived from man (Marie, 1994) or rodents (Rodan *et al.* 1994) may well differ from those of normal cells, depending on the nature of the genetic transforming event (Rodan & Noda, 1991). These limitations can be minimised by using permanent cell lines immortalised with specific oncogenes, such as the large T antigen genes of viruses like SV-40 and polyoma. Cloning offers the advantage that the phenotype of a cell population can be traced back to a single progenitor cell. Although these cell lines offer advantages over primary culture and osteosarcoma culture systems, they also have limitations. A major caveat is that the phenotype of cells in long-term culture may reflect adaptations to the *in vitro* environment as well as the properties they possessed *in vivo*. Because even clonally derived cells are phenotypically heterogeneous and may undergo phenotypic drift with time, they need to be checked periodically for phenotypic features.

Osteoblast features in cell culture. Osteoblasts are the bone-forming cells of the skeleton; they synthesise

and regulate the deposition and mineralisation of the extracellular matrix of bone. Osteoblasts may be identified in intact tissue either *in vivo* or in organ culture by their biochemical and morphological parameters (Rodan & Rodan, 1984). They contain the mRNA coding for molecules produced by osteoblasts *in vivo*, such as bone matrix constituents, enzyme markers, and they respond to calciotropic hormones. Morphological criteria include their location in bone tissue, spatial relationships with respect to their matrix and other cells, and staining characteristics indicating the active production of extracellular matrix. Both biochemical and morphological assays have been used to assess osteoblast activity in cultured cells.

Biochemical characteristics and assays. The regulation of osteoblast proliferation and differentiation involves the sequential expression of cell growth and tissue-specific genes in response to a series of regulatory signals mediated by physiological factors that include, but are not restricted to, growth factors, polypeptide and steroid hormones, and morphogenes. The three principal periods of the development are proliferation, matrix development and maturation, and mineralisation. Type I collagen–fibronectin is synthesised during the proliferation period, and then continues to mature and mineralise. The formation of this matrix inhibits proliferation, and matrix mineralisation inhibits the expression of genes associated with matrix maturation. Phenotype genes, such as those for osteocalcin and alkaline phosphatase, are not expressed during the growth period. Apoptosis occurs in mature nodules, and is associated with turnover of the matrix mediated by collagenase activity and focal compensatory proliferation (Stein *et al.* 1996).

The control of osteoblast growth must support competency for proliferation and cell cycle progression within several biologically defined contexts. However, the isolation of osteoblasts from *in vivo* tissue environments and transfer to *in vitro* culture results in their proliferation. Post-confluent proliferation subsequently leads to focal multi-layering of bone-forming cells and the synthesis of type I collagen that establishes a bone tissue-like organisation. Limited proliferation occurs in mature, mineralising bone nodules together with apoptosis and increased collagenase activity (Stein *et al.* 1996).

Early biochemical studies used traditional methods of enzyme assay or structural analysis, but the recent availability of immunological and genetic probes for many of the bone matrix proteins and other osteoblast-associated molecules allows the assay of multiple phenotype markers in cells in culture.

One of the most frequently assayed biochemical markers is alkaline phosphatase, which is simple to measure biochemically (Sodek & Berkman, 1987). Its expression pattern in osteoblasts (Doty & Schofield, 1976; Stein *et al.* 1996) and its involvement in mineralisation have been extensively documented (Wuthier & Register, 1984). However, many cell types in bone or marrow stroma contain alkaline phosphatase, such as hypertrophic chondrocytes (Wuthier & Register, 1984) and adipocytes (Beresford *et al.* 1993), while fibroblastic cells also have a low concentration of this enzyme. The alkaline phosphatase activity can provide a reasonable indicator of osteoblast cells if the cartilage and marrow are removed by dissection.

A second indicator of osteoblast activity is the production of bone matrix molecules, the most abundant of which is type I collagen. Classical methods assess collagen production by measuring the protein (Sodek & Berkman, 1987). The percentage of total protein synthesis due to collagen can be determined by radiolabelling and SDS electrophoresis. This separation of proteins is very useful since it can identify collagen other than type I, although this collagen seems to be the only type produced by mature osteoblasts. This method also provides an estimate of fraction purity, since the presence of type II collagen could indicate the presence of chondrocytes and type III collagen the presence of fibroblasts. Like type I collagen and alkaline phosphatase, many molecules of bone matrix, including osteonectin and osteopontin (Nomura *et al.* 1988), osteocalcin (Fleet & Hock, 1994) and thrombospondin (Aubin *et al.* 1995), can be considered to be indicators of the osteoblast phenotype, although they are not produced by osteoblasts exclusively. Specific mRNAs have been measured to assess expression of osteoblast-associated genes as the corresponding genetic probes have become available. One advantage of this technology is that a single total RNA preparation from a cell culture may be used to assay several markers by Northern blot analysis. The recent development of methods based on the reverse transcriptase–polymerase chain reaction has further extended the flexibility of these assays by allowing analysis of extremely small numbers of cells (Aubin *et al.* 1995). The limitations of these techniques are those inherent in the methods themselves, such as the need for appropriate controls and adaptations of quantitative analysis.

A third biochemical indicator of the osteoblast phenotype is responsiveness to calciotropic hormones like parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D_3 (calcitriol). PTH-stimulated cyclic AMP production was one of the earliest phenotypes analysed in cultured bone cells, and this property was later linked with several phenotypic responses to PTH by osteoblast cells (Rodan & Rodan, 1984). The presence of PTH-dependent adenylate cyclase may not, however, be sufficient to define an osteoblast cell. The responses of cells to osteotropic agents may also include the modulation of alkaline phosphatase activity by PTH (Rodan *et al.* 1994) and the stimulation of osteocalcin (bone gla-protein) synthesis by calcitriol (Owen *et al.* 1990). These agents often produce complex, biphasic effects on the osteoblast phenotype, depending on the stage of the culture (proliferation and differentiation; Owen *et al.* 1990; Rodan *et al.* 1994).

Morphological assays. Morphological assays can provide complementary information since biochemical changes in phenotype may occur focally rather than uniformly throughout the culture. Histochemical staining for alkaline phosphatase is often used as a simple screening test to differentiate presumptive osteoblast cells from fibroblasts (Doty & Schofield, 1976). Immunohistochemistry and *in situ* hybridisation can help to detect various phenotypic markers of osteoblasts, particularly the matrix constituents.

Osteogenic potential assays. As the ultimate product of osteoblasts is bone, the primary test of the osteoblast phenotype in cultured cells is the production of an organised, mineralised tissue that resembles bone as closely as possible. Both *in vivo* and *in vitro* methods have been used to test the osteoblastic nature of cultured cells.

In vivo diffusion chambers with filters allowing the flow of macromolecules and nutrients, but preventing the passage of cells and direct cellular interactions, are routinely used. These chambers containing the cells are placed subcutaneously or intraperitoneally in host animals, and examined histologically after several days or weeks for the formation of bone (Nakahara *et al.* 1990). This allows cells cultured *in vitro* to be exposed to an *in vivo* environment and their assay by full histological analysis. However, this will not determine whether the cells in culture already had osteoblast characteristics or were precursors that underwent osteoblast differentiation whilst in the chamber. It is also difficult to establish a quantitative relationship between the numbers of osteogenic cells in a population and the tissue formation response.

In vitro assays have been used for cells cultured as monolayers. The culture medium is usually supplemented with ascorbic acid, needed for collagen hydroxylation, and β-glycerol phosphate, an organic phosphate whose presence facilitates mineralisation (Ecarot-Charrier *et al.* 1983; Peck *et al.* 1984; Bellows *et al.* 1990; Quarles *et al.* 1992; Beresford *et al.* 1993). Under these conditions, the cells begin to form nodules composed of multiple layers of alkaline phosphatase-enriched cells and intercellular matrix. This system is suitable for several types of investigation, and has been used for biochemical and morphological investigations of differentiated osteoblasts (Ecarot-Charrier *et al.* 1983; Peck *et al.* 1984; Bellows *et al.* 1990; Quarles *et al.* 1992; Beresford *et al.* 1993). Cells grown in these systems mimic the morphological and biochemical features that occur during osteogenesis *in vivo* (Ecarot-Charrier *et al.* 1983; Wuthier & Register, 1984). Nevertheless, these systems that use organic phosphates to promote mineralisation raise some questions about the validity of such an osteogenic process. There are no clear indications of high concentrations of organic phosphates *in vivo*, and mineralisation occurs in some cell systems without exogenous organic phosphate (Satomura *et al.* 1991).

Primary and early passage cultures. Many cell culture models use cells isolated from the bones of fetal or neonatal mice (Wong & Ng, 1992), rats (Luben *et al.* 1976), chickens (Nijweide *et al.* 1981), pigs (Denis *et al.* 1994) or cattle (Whitson *et al.* 1992) and grown in primary cultures or for a few passages. Tissues from such young animals are excellent sources of cells because they proliferate rapidly *in vitro*, and the rapid growth of bone during this phase of life ensures that the bone contains a high proportion of active, phenotypically mature osteoblasts.

Calvaria (particularly parietal bones) are easy to dissect out, contain little bone marrow and are not yet fully mineralised. Cells are generally released by digestion of the calvaria with proteases like collagenase or trypsin (Luben *et al.* 1976; Peck *et al.* 1984; Wong & Ng, 1992). Populations enriched in osteoblast cells have been obtained by fractionation techniques including density separation (Wong & Ng, 1992), and the selection of some populations during the enzymatic digestion of the bone (Luben *et al.* 1976). Cells from the later digests (after 40–80 min) have a more osteoblast phenotype than those released first (Luben *et al.* 1976). Cells isolated in this way have become one of the standard osteoblast culture systems. These cells show all of the biochemical traits of osteoblasts and mineralisation when placed in monolayer culture (Ecarot-Charrier *et al.* 1983; Rodan & Rodan, 1984; Bellows *et al.* 1990). Although enzyme digestion has also been used to obtain osteoblast-like cells from mineralised adult bone, including the endosteal bone of rabbits (Yee, 1983) and adult human bone (Evans *et al.* 1990), the more commonly used technique is outgrowth from explants of mature human bones obtained at biopsy or surgical resection. This method is more time-consuming than the digestion technique, but involves less damage to the cells from proteases (Robey & Termine, 1985; Marie, 1994; Robey, 1995). Explants can also be cultured in Ca-low media to select osteoblast-like cells from fibroblasts (Robey & Termine, 1985). Although the precise identity of the cells that proliferate and grow out from bone explants has not yet fully established (e.g. osteocytes or lining cells; Robey, 1995), these cells have the full range of osteoblast features in culture but cannot produce a mineralised matrix with a bone-like structure *in vitro*.

Not all of the morphologically identifiable cell types can yet be identified and separated clearly. The development of antibodies recognising specific bone cell types may solve this problem. Immunoselection with monoclonal antibodies specifically directed against osteoblasts (van der Plas *et al.* 1994; Walsh *et al.* 1994) has been used to isolate and culture chick bone osteocytes (van der Plas *et al.* 1994) that are morphologically different from osteoblasts in culture. Human bone cell cultures also have a number of limitations. The lack of osteogenic potential of the cells *in vitro* may be due to the absence of an appropriate factor, because transplanted human bone-derived cells are osteogenic *in vivo*. The osteoblast phenotype may vary widely from one donor to another, due, in part, to uncontrolled culture conditions. Because the osteoblast characteristics of the cells decrease rapidly with the number of passages and the time in culture (Marie *et al.* 1989), only primary cultures or first-passage cells should be used. Bone cells must also be obtained from age-matched subjects to allow a valid comparison between normal and pathological cell populations, because the osteoblast characteristics of human bone-derived cells may vary with the age of the donor (Evans *et al.* 1990; Fedarko *et al.* 1992; Katzburg *et al.* 1999). Lastly, human bone cells tend to produce heterogeneous cell cultures, perhaps because of the presence of mixed cell populations with different growth rates or cells at various stages of differentiation (Matsuyama *et al.* 1990).

Permanent cell lines. The earliest osteoblastic cell lines were isolated from rat osteosarcomas. Two cell lines were established in the laboratories of Rodan and Martin based on the PTH-responsive adenylyl cyclase system (Partridge *et al.* 1983; Rodan *et al.* 1994). The clonal ROS cell lines (Rodan *et al.* 1994) were derived from a spontaneous

tumour in an ACI (August with Irish cort × Copenhagen 2331) rat, and the UMR cell lines were isolated from [^{32}P] orthophosphate-induced tumours in rat (Partridge *et al.* 1983). The most widely used osteosarcoma cell lines, UMR-106, ROS 17/2 and ROS 17/2·8, all respond to PTH and have many other osteoblast traits, but they differ phenotypically from normal cells. Several cell lines isolated from human osteosarcomas, such as SaOS (Rodan *et al.* 1987), TE-85 (Clover & Gowen, 1994), MG-63 (Billiau *et al.* 1977) and OHS-4 (Fournier & Price, 1991), have been used. Osteosarcoma cell lines have been used mostly for testing the synthesis of bone matrix proteins. Only a few of these human osteosarcoma cell lines, e.g. SaOS and OHS-4, have been characterised extensively for their osteoblast features. One major limitation is the loss of osteoblast characteristics after a number of passages.

Permanent osteoblast-like cell lines have been established from normal bone. Early clonal lines, such as the RCJ cell lines from normal rat calvaria (Aubin *et al.* 1982) and the clonal MC3T3-E1 cell line from normal mouse calvaria (Sudo *et al.* 1983), have been isolated from rodent tissue. One of the RCJ cell lines gives multiple phenotypes in culture, including cartilage, muscle and fat cells. Sub-clones of clonal cell lines from rat calvaria explants tend to differentiate *in vitro* into muscle and adipocytes (Yamaguchi & Kahn, 1991). The MC3T3-E1 line was isolated by selecting an alkaline-rich clone from a population of cells maintained under specific sub-culture conditions. This cell line appears to resemble osteoblast-enriched calvarial cells in that the osteoblast phenotype does not develop during the proliferative stage but increases when the cells become confluent (Quarles *et al.* 1992).

Cell lines established from periosteum and bone marrow have also been used to investigate osteoblast differentiation. A non-transformed, non-immortalised cell line with a limited life span (UMR-201) from the rat periosteum has been used to demonstrate the induction of osteoblast features by retinoic acid (Ng *et al.* 1988). Cell lines from bone marrow have also provided osteoprogenitors for bone formation and to maintain a suitable environment for haematopoiesis. The clonal marrow stromal cell line MBA-15 with osteogenic properties produces a variety of factors, which modulate myeloid and lympoid cell growth (Benayahu *et al.* 1991). A series of clonal lines, which have the phenotypes of pre-adipocytes, fibroblast or endothelial-like cells, may also have osteoblast features (Benayahu *et al.* 1992).

Permanent cell lines derived from normal bone tissue by spontaneous immortalisation events suffer from a lack of information about the nature of this event and the possible effects on phenotype.

Osteoblast cell lines have now been established by characterised immortalisation mechanisms. Cells are immortalised by transfection with the large T antigen of SV-40 (Jat & Sharp, 1986). In conjunction with a selectable antibiotic resistance gene, this technique has been used to generate two cell lines from rat calvarial cells (Heath *et al.* 1989). One line, RCT-3 selected from the late digest population, was found to be constitutively osteoblastic, whereas the RTC-1 cell line, established from the early digest population, expressed osteoblast features only after induction by retinoic acid. Another oncogene, *myc*, has also been used to immortalise calvarial cells (Hofstetter *et al.* 1991). Several human osteoblastic cell lines have also been immortalised with similar techniques. These include the HOBIT cell line from human bone cells from a 68-year-old woman (Keeting *et al.* 1992), the hFOB cell line from fetal human bone immortalised with a temperature-sensitive mutant of SV-40 large T antigen (Harris *et al.* 1995), and the AHTO-7 cell line from adult trabecular osteoblast AHTO cell line (Lomri *et al.* 1999). Another method for establishing osteoclastic cell lines can also be used for immortalised osteoblast cell lines (Chambers *et al.* 1993). Both stromal and haematopoietic cell lines were established from a transgenic mouse bearing the temperature-sensitive SV-40 large T antigen gene. This technique allows *a priori* the evaluation of the effects of an immortalising transgene on the development of tissue *in vivo*.

Osteoprogenitor cell cultures. The bone cell culture systems described above were used to study the functions and regulatory mechanisms of mature osteoblasts, e.g. organisation, synthesis, secretion and mineralisation of the extracellular matrix. Many recent bone cell culture systems have focused on using osteoblast progenitor cells to study their developmental osteogenic potential, their relationships with other cell lineages, including chondrocytes, myoblasts, fibroblasts and adipocytes, and the factors that influence their differentiation to osteoblasts or other cell types.

Osteogenic cells are present in the stroma of mammalian bone marrow, and their ability to produce bone-like mineralised tissue has been demonstrated both *in vivo*, in diffusion chambers loaded with bone marrow cells (Friedenstein, 1990), and *in vitro*, where bone-like tissue is synthesised by various marrow stromal cell populations under suitable conditions (Benayahu *et al.* 1992; Malaval *et al.* 1994). These studies indicate that most of the osteoprogenitors in stromal cultures seem to be of the relatively immature osteoblasts.

Bone marrow stroma also contains other cell types in addition to osteoprogenitors, including fibroblasts, adipocytes and endothelial cells (Benayahu *et al.* 1992). The term fibroblast refers not only to cells involved in forming the connective tissue, but also describes a general category of undifferentiated bone marrow stromal cells. The term fibroblast colony-forming unit refers to colonies associated with a mixture of cell types. Haematopoietic cells are closely related to stromal cells in bone marrow, but most well-differentiated haematopoietic cells are normally removed from stromal cell cultures by selective washing and adherence techniques (Bellows *et al.* 1990). Limiting dilution analysis of osteoprogenitor cells in bone marrow cultures has indicated that only one cell type is limiting in such cell populations for osteoprogenitor cell expression (Gurdon *et al.* 1993). This raises two possibilities: one is that establishment of a group of differentiated osteoblasts may be independent of the cell interactions that occur only when a critical number of cells is reached (Aubin, 1999); the second is that heterotypic cell–cell interactions occur, such that osteogenesis is regulated by non-osteogenic

lineages in the bone marrow (Zhang *et al.* 1991). In other respects, osteogenesis was stimulated at the same time as the formation of more bone nodules when a large number of cells from the non-adherent fraction of bone marrow was added to stromal cultures. This suggests that there is a class of osteoprogenitors in the non-adherent fraction of bone marrow (Long *et al.* 1995; Malaval *et al.* 1999).

Osteoclast formation and bone resorption studies

Bone resorption plays an important role in pathological disorders such as osteoporosis, metabolic bone diseases, fractures and malignant hypercalcemias, in addition to its physiological role. Osteoclasts are the primary cells responsible for bone resorption, and elucidation of how the osteoclasts are regulated is essential to an understanding of the whole process of bone metabolism. But it is difficult to obtain information about osteoclasts because it is difficult to isolate these cells. They are very few osteoclasts in bone tissue ($<0{\cdot}01\,\%$), and osteoclasts are fragile and adhere tightly to the calcified bone matrix. Osteoclasts are post-mitotic multinucleated cells formed by fusion of their progenitors, and few osteoclast cell lines are available.

The key molecules that regulate osteoclast recruitment and function have recently been identified. The signal that links osteoblasts/stromal cells to osteoclast precursors is a protein on the osteoblast/stromal cell membranes, a member of the tumour necrosis factor ligand family, the receptor activator of NF-κB (RANK) ligand (RANK-L; American Society for Bone and Mineral Research President's Committee on Nomenclature, 2000). Osteoclast progenitors bear RANK and recognise RANK-L through cell-to-cell interaction. Osteoprotegerin can also bind to RANK-L, inhibiting osteoclast formation and activity (Suda *et al.* 1999). Osteoblasts and stromal cells can now be replaced by RANK-L and M-CSF in cultures of haematopoietic cells (Sells Galvin *et al.* 1999).

Authentic functional osteoclasts are needed to examine the mechanisms of bone resorption. Each experiment requires osteoclasts to be isolated from bone or generated *in vitro* from their haematopoietic progenitors. Several approaches, including bone resorption assays, have been developed to study osteoclast biology and function over the past decade. Congenital mutations and transgenic mice have also provided insights into regulatory systems that cannot be modelled *in vitro*.

Isolation of osteoclasts from bone

Fully differentiated osteoclasts may be isolated from bones that differ in the purity and yield of cells they provide.

Osteoclast isolation from newborn rat or rabbit long bones. The bones are minced in culture medium, and the supernatant collected, seeded onto glass, plastic or bone slices, and incubated for 20–30 min. The non-adherent cells are removed, and the osteoclasts, which remain on the substratum, are further incubated for the desired period. This method, based on the adherence of osteoclasts to the substratum, is widely used in single-cell electrophysiological, immunocytochemical and histochemical studies, because of the impurity and insufficient number of osteoclasts obtained (Chambers & Magnus, 1982; Chambers *et al.* 1984; Tezuka *et al.* 1992).

Larger numbers of rabbit osteoclasts can be obtained by seeding the cells on collagen-coated discs. The non-adherent cells are removed, and osteoclasts are released by digestion with dilute collagenase. This method again depends on the strong adherence of rabbit osteoclasts to collagen. It is suitable for the estimation of mature osteoclast bone-resorbing activity because osteoclasts can be cultured with controlled cell number and purity, but is not applicable to rodent or human osteoclasts.

Osteoclast isolation from chick long bones. Medullary bones of laying hens fed a Ca-deficient diet are minced into culture medium and digested with collagenase. Osteoclasts may be enriched by filtering (Zambonin Zallone *et al.* 1982; de Vernejoul *et al.* 1988), by discontinuous density centrifugation (Oursler *et al.* 1991) or by using magnetic beads coated with monoclonal antibodies raised against avian osteoclasts (Collin-Osdoby *et al.* 1991).

Osteoclasts from giant cell tumours. The giant cells from these tumours are highly activated osteoclasts that can form resorption lacunae on calcified matrices. They have been used by several groups as a model for human osteoclasts (Chambers *et al.* 1985; Grano *et al.* 1994).

Osteoclast-like cells from bone marrow cultures. Since osteoclasts are haematopoietic in origin, belonging to the monocyte–macrophage lineage, bone marrow culture techniques have been used to study osteoclast formation. The osteoclast-like cells that differentiate in these cultures are fully mature and functional, and satisfy the major criteria for osteoclasts, including multinuclearity (>2 nuclei), tartrate-resistant acid phosphatase (TRAP), calcitonin receptor, $pp^{60c\text{-}src}$ expression, vitronectin receptor and, the main osteoclast phenotype, the ability to resorb bone.

Marrow culture systems that form osteoclast-like cells include mouse and pig marrow, in which TRAP-positive multinucleated cells able to resorb bone form in 5–6 d in the presence of calcitriol (Takahashi *et al.* 1988; Rassi *et al.* 2002), and human bone marrow, where active osteoclasts develop after 4 weeks in culture in the presence of calcitriol and M-CSF (Udagawa *et al.* 1989; Sarma & Flanagan, 1996). Osteoclast-like cells are found near colonies of alkaline phosphatase-positive cells in these cultures, indicating the involvement of osteoblasts or bone stromal cells in osteoclastogenesis. In fact, bone marrow contains not only haematopoietic osteoclast progenitors but also many stromal cells and osteoblasts, and cell-to-cell contact is absolutely required for osteoclast differentiation.

Mouse spleen cells have been used as these cells differentiate into osteoclast-like cells when co-cultured with appropriate bone marrow-derived stromal cells or osteoblasts (Udagawa *et al.* 1989). Osteoclast-like cells have also been isolated from spleen cells cultured on collagen gels. This method allows the production of large numbers of osteoclasts suitable for biochemical or molecular biological studies (Akatsu *et al.* 1992).

Pre-fusion osteoclasts may be isolated from similar cultures by treatment with echistatin. This method is based on the fact that osteoclasts and osteoclast progenitors

bear abundant amounts of the vitronectin receptor, $\alpha_v\beta_3$, on their membranes. Echistatin is an arginyl–glycyl–aspartyl-containing snake venom that binds strongly to $\alpha_v\beta_3$ and inhibits the attachment of osteoclast cells (Wesolowski *et al.* 1995).

Osteoclast precursors and osteoclast precursor cell lines

Mammalian precursors in the early stages of differentiation contain TRAP, and later acquire calcitonin receptors, vitronectin receptor and $pp^{60c\text{-}src}$.

Several haematopoietic cells and cell lines, grown in the presence of osteoblast/stromal cells, growth factors and cytokines, have been used to obtain osteoclast-like cells from their progenitors. These have included mature murine monocytes or macrophages (Udagawa *et al.* 1990), immortalised murine macrophage-like cells (Miyamoto *et al.* 1998), avian monocytic cells (Solari *et al.* 1996), human myelo-monocytic leukaemia cells (Gattei *et al.* 1992), mononuclear cells from giant cell tumours (James *et al.* 1996) and human peripheral blood mononuclear cells (Matsuzaki *et al.* 1998). Fully functional osteoclast-like cells develop from the rodent cultures, whereas multinucleated cells from the avian and the human systems have several osteoclast characteristics, but do not regularly resorb bone.

Clonal analysis of haematopoietic cells by surface phenotypes has been used to further identify osteoclast precursors by characterising osteoclast-like cells distinct from other haematopoietic progenitors (Lee *et al.* 1992*b*; Muguruma & Lee, 1998). Surface phenotype analysis has also shown that human osteoclasts are derived from CD14+ monocytes (Massey & Flanagan, 1999).

Bone resorption assays

These assays can be used to examine osteoclast resorptive activity *in vitro*, including tissue organ culture, in which individual bones are cultured almost intact, and osteoclasts cultured on resorbable substrates, bone particles and bone or dentine slices.

Tissue organ culture. Organ cultures rely upon the release *in vitro* of Ca from long bones or calvaria from newborn mice or rats (Raisz, 1963; van der Pluijm *et al.* 1994; Most *et al.* 1995). The bones are pre-labelled by incorporation into the pups of ^{45}Ca or [^{3}H]proline injected into the pregnant mother. The release of the isotopes from the cultured bones is measured at the end of the experiment, which can be continued for at least 5 d. This assay has been used frequently, but the cell composition of explants is extremely heterogeneous. It is also difficult to identify target cells, and to distinguish between direct and indirect effects on osteoclasts.

Bone particle-based assays. Bone particles, obtained by grinding of ^{45}Ca- or [^{3}H]proline-labelled bones, are cultured with osteoclasts. ^{45}Ca or [^{3}H] release is measured at the end of the cultures (Oreffo *et al.* 1988).

Bone slice assay. This widely used assay is based on the observation that isolated osteoclasts make resorption pits on slices of devitalised dentine or bone (Boyde *et al.* 1984) and that these pits resemble Howship's lacunae. Basal resorption by osteoclasts depends on the pH of the culture medium, which can be manipulated by altering the concentration of HCO_3^- or CO_2. A low pH of 6·8–7·2 stimulates resorption by neonatal rat osteoclasts (Murrills *et al.* 1993; Arnett *et al.* 1994). The osteoclasts are incubated on the slices for 1–2 d, and the cells removed mechanically or by sonication. The pits are stained to detect the organic fringe at the border of the pits by reflected light or scanning electron microscopy. The extent of the resorption is usually measured by counting the number and/or by measuring the area of the pits (Murrills *et al.* 1990).

Congenital mutations and transgenic mice. Studies on congenital osteopetroses have established the haematopoietic origin of osteoclasts. These diseases have contributed to a better understanding of several essential mechanisms of bone resorption, including the role of carbonic anhydrase II (Sly *et al.* 1983) and the M-CSF gene (Yoshida *et al.* 1990). Techniques of homologous recombination have demonstrated the role of the *c-fos* gene (Grigoriadis *et al.* 1994), and targeted disruption of the *c-src* gene results in osteopetrosis, showing that $pp^{60c\text{-}src}$ PTK is essential for osteoclast activity (Soriano *et al.* 1991). The TRAP promoter has also used to target the *bcl*-X_L and/or SV-40 large T antigen to the osteoclast lineage of transgenic mice, as a way of immortalising osteoclast precursors (Hentunen *et al.* 1998). This allows a much more effective immortalisation of progenitors that form osteoclast-like cells than those in normal bone marrow.

Genomics and proteomics techniques

Many of the genomics techniques used to gain information on the oestrogenic properties of phyto-oestrogens have already been referred to in this review. In recent years, however, proteomics has emerged as a more functional, complementary technique for identifying proteins associated with specific changes in a metabolic pathway (Service, 2000).

Genomics and gene microarrays

A wide range of methods has been used to determine the genes targeted by phyto-oestrogens in specific cell types and to identify those tissues most directly affected. The phenotypes of transgenic mice lacking either ERα or ERβ have provided valuable information on the most susceptible targets. Thus ERβ 'knockout' mice have particular defects in bone metabolism not encountered in their ERα 'knockout' counterparts. Recently, more refined techniques using reporter genes under the control of one or more ER elements have been used to identify the targets for phyto-oestrogens in tissues and in specific cell types. Once the relevant ER is activated, expression of the reporter gene, which is often β-galactosidase, can be readily detected using a colorimetric assay.

A series of differential display techniques has been available for many years. In these, the changes in gene expression caused by a specific component can be monitored and the genes identified by sequence analysis. Gene expression can now be directly visualised by microarray.

Arrays containing a relatively small number of genes in particular metabolic pathways are available, as well as more complete arrays containing more than 10 000 genes.

Proteomics and phage display

Improved automation and robotic techniques, together with integration with bioinformatic systems and more complete databases, have made it possible to separate many thousands of proteins by two-dimensional electrophoresis. The components can then be identified by MS of peptides produced by proteolytic digestion (usually trypsin). The use of differential display has been facilitated particularly by the introduction of several fluorescence labelling methods. The proteins induced (or inhibited) by a specific phyto-oestrogen can thus be visualised and identified. One problem at present is that many proteins are still unknown and cannot easily be identified by current methods. Conventionally, larger amounts of the unknown protein must be isolated for amino acid sequence analysis. This may often involve an intermediate step of producing monoclonal antibodies to facilitate the isolation of larger quantities of the protein. The use of antibody libraries expressed on phage (McCafferty *et al.* 1990) may well provide a rapid solution to this problem. The electrophoretogram can be screened with the library, and the phage antibody interacting with the components of interest can be excised and expanded to produce specific antibodies (Liu & Marks, 2000). The antibody libraries currently available are relatively large, containing 10^9 to 10^{12} distinct single-chain or F_{ab} fragment antibodies, so that there is a good chance of obtaining useful antibodies with high affinity.

These newer techniques combining genomics with proteomics and phage display offer unique capabilities for identifying the targets for phyto-oestrogen action and will, therefore, contribute to a better understanding of the mechanisms involved.

Conclusions and recommendations

Clearly, elucidation of the effects of phyto-oestrogens on bone cells using *in vitro* experiments is a complex area, which is further complicated by the multi-factorial influences on cell function. Nevertheless, it is possible to identify some general principles that must be taken into account in developing further experiments.

1. Organ culture systems have some advantages over cell cultures as they make possible appropriate cell–cell and cell–matrix interactions.
2. The use of well-characterised cell lines ensures homogeneous response from osteoblast cell cultures. The results obtained should be confirmed, wherever possible, using primary osteoblast cell cultures.
3. It is essential to determine the ER status of all cell culture systems, to enable adequate interpretation of the results.
4. Marrow cultures and spleen stromal cell cultures appear to be the best model systems for studying osteoclast formation, and isolated osteoclasts are better for testing the effects of factors on mature osteoclasts.

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